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Two conserved tryptophan residues of tumor necrosis factor and lymphotoxin are not involved in the biological activity

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Each of the two highly conserved tryptophan residues in hTNF (positions 28 and 114) was converted into phenylalanine by site-directed mutagenesis and the mutant proteins were partially purified. A cytotoxicity assay on mouse L929 cells showed only a slight reduction in biological activity, strongly suggesting that neither of the two amino acids is involved in the active site.

Tumor necrosis factor; Site-specific mutagenesis; Cytotoxicity

1. INTRODUCTION

Human and murine tumor necrosis fractors (hTNF and mTNF), products derived from macrophages, exist as trimers consisting of three equal subunits of 17 kDa [1,2]. The monomers have a length of 157 and 156 amino acids and show a homology of 79%. Both contain an unusually long prepeptide of 76 and 79 amino acids respectively, with an even higher degree of amino acid conservation of 86% [3]. The amino acid sequence of the rabbit TNF (rTNF) revealed a monomer of 154 amino acids with 80% homology, as compared with hTNF. Lymphotoxin (LT), also referred to as hTNF-β, a related lymphokine produced by lymphocytes, is preceded by a classical signal peptide [4]; the mature human and mouse contain 172 and 169 amino acids, giving a homology of 28 and 31%, respectively, with the corresponding TNF [5,6].

Comparing these 5 related sequences, four strongly conserved regions can be deduced (positions 11–15, 48–64, 119–133, 152–157; hTNF numbering) (fig.1). TNF contains a central region which has no similarity to the lymphotoxins and is almost completely enclosed between the two conserved cysteine residues (positions 69, 101). Cys 69 can still be found in mLT. These cysteine residues in TNF form a disulfide bridge, that is probably not necessary for maintaining the overall conformation as shown by CD spectra [7,8]. Fluorescence spectra and quenching, however, indicated that, although the biological activity was not reduced significantly, the local conformation around the two tryptophan residues was affected after removing this disulfide bond [9].

Our attention was attracted by these two tryptophan residues which are highly conserved among all the molecules sequenced so far. Here, we evaluate whether this conservation is related to their functional importance, or whether these residues play a merely structural role. We converted each tryptophan residue by site-specific mutagenesis into phenylalanine (Phe) and subsequently examined the effect on the cytotoxic activity of hTNF for malignant cells. It was found that the two mutant proteins, 28F and 114F, only exhibit a somewhat reduced specific activity: 45 and 30% of wild type, respectively. This indicates that these residues are not involved in the active site.

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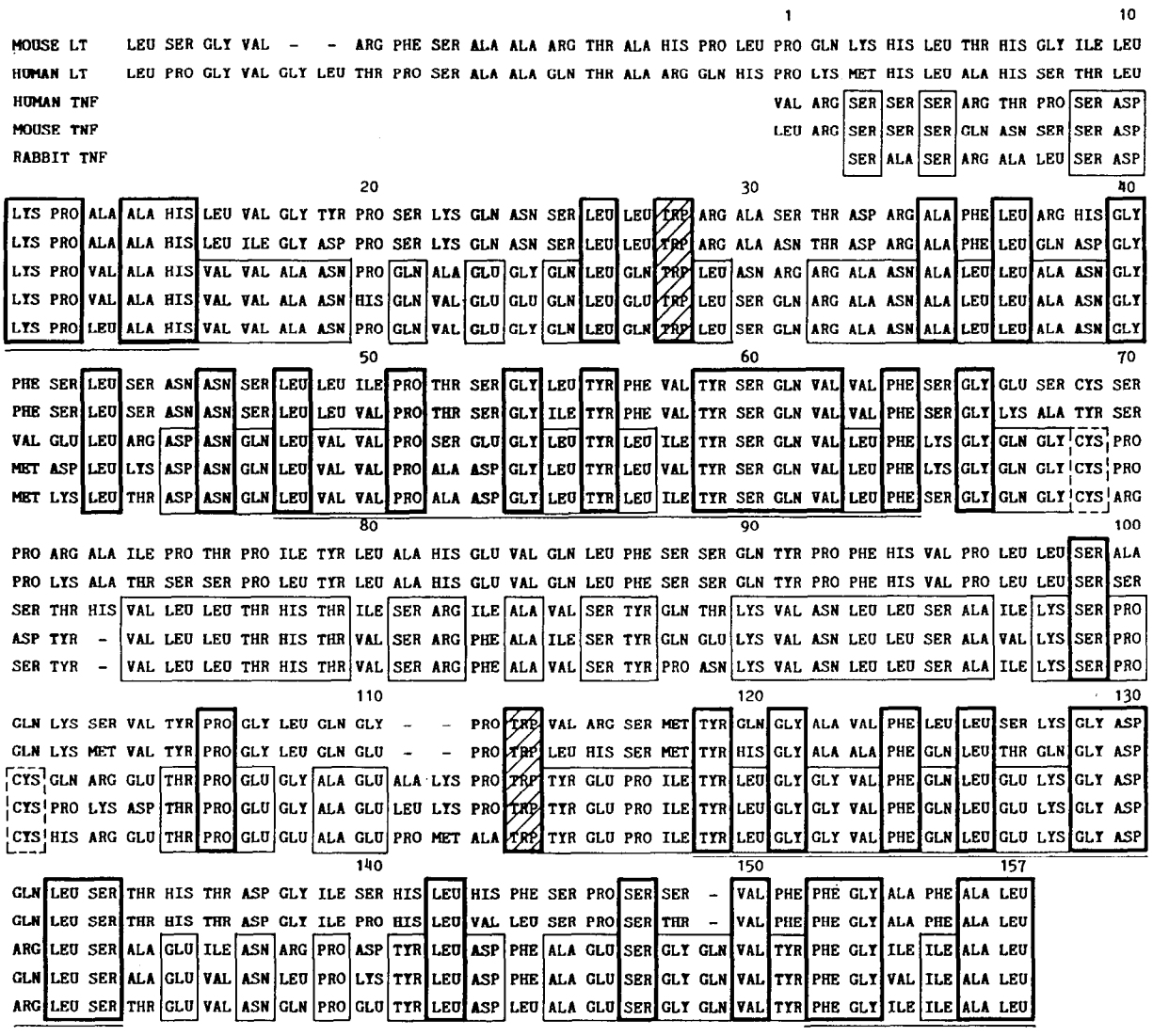

Fig.1. Amino acid sequence comparison of TNFs and LTs. Numbering refers to the mature hTNF sequence. Homology between TNFs, or between TNFs and LTs is represented by fine and bold boxes, respectively, Cys residues by dashed boxes and the two strongly conserved Trp residues by hatched boxes. The four strongly conserved regions are underlined. Adapted from Tavernier et al. [5].

2. MATERIALS AND METHODS

2.1. *Insertion of the hTNF gene into M13 mp11 DNA*

The entire hTNF gene, coupled to the tryptophan A terminator sequence (TA), was inserted into the M13 mp11 vector which was opened by *Eco*RI and *Hin*dIII. This was done using an *Eco*RI-*Bst*E_2 fragment (from the pP_LC-mu236-hTNF1 plasmid [3]) and the *Bst*E_2-*Hin*dIII fragment from the pP_L-T_4-hTNF-T vector (fig.2).

2.2. *Site-specific mutagenesis of the recombinant hTNF gene*

Mutagenesis of the two tryptophan residues was performed using a selection method based on uracil-containing single-stranded templates of M13 DNA [10]. Hybridization was performed with oligonucleotides containing the proposed mutations and removing or introducing a restriction site (fig.3, see also section 3).

2.3. *Subcloning of the mutant genes into an expression vector and control of expression*

The genes containing either the 28F or 114F mutations were transferred as *Ava*I-*Hin*dIII fragments from the M13 vector to the *Ava*I-*Hin*dIII-opened pMa-T_4-hTNF-TA vector (fig.2), resulting in the phasmids pMa-T_4-hTNF28FTA or pMa-

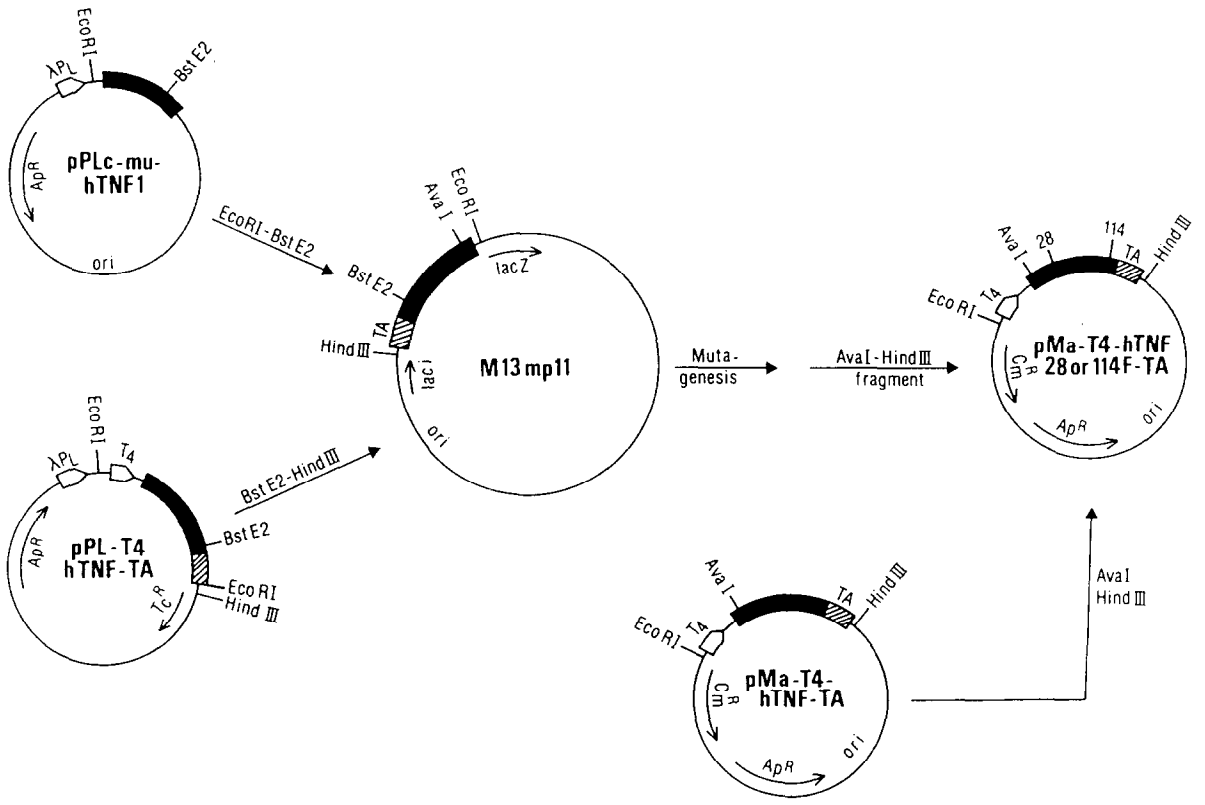

Fig.2. Insertion of the wild-type hTNF gene into the M13 mp11 vector from the plasmids pP_{LC}-mu236-hTNF1 and pP_L-T_4-hTNF-TA followed by insertion of the mutant genes into the pMa-T_4-hTNF-TA expression vector. Only relevant restriction sites are shown. The hTNF cDNA information is indicated as a filled-in bar. Open arrows represent promoters; the hatched box is the Trp A terminator sequence. Ap^R, ampicillin resistance; Tc^R, tetracyclin resistance; Cm^R, chloramphenicol resistance.

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                                        Trp28
hTNF          GAG  GGG  CAG  CTC  CAG  TGG  CTG  AAC  CGC  CGG  GCC  AAT
28F PROBE          G  CAG  CTG  CAG  TTC  CTG  AAC  CGC  CGG  G
(26-mer)              Pvu2         Phe
                           Pst1

                                   Trp114
hTNF          GAG  GCC  AAG  CCC  TGG  TAT  GAG  CCC  ATC  TAT  CTG  GGA GGG
                           BstN1
                           ScrF1
114F PROBE         CC  AAG  CCC  TTC  TAT  GAG  CCG  ATC  TAT  CTG  G
(32-mer)                    Phe            Sau3A
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Fig.3. Oligonucleotide sequences used for mutagenesis and screening of mutants. Differing bases appear in bold on the probe sequence. Disappearing and appearing restriction sites are underlined on the wild-type hTNF sequence and the oligonucleotide sequence, respectively. The Trp and Phe codons are indicated by horizontal square brackets.

T_4-hTNF114F-TA, and transformed to *E. coli* MC1061. The pMa phasmid was originally constructed for generating ssDNA of an inserted gene; it can however, also be used as an expression system [11]. To test the solubility and expression level, 1 ml culture was sonicated and centrifuged. The pellet, containing the insoluble fraction, was dissolved in 100 μl Laemmli buffer [12] whereas the supernatant was precipitated with 5 vols ethanol and spun down. The resulting pellet was also redissolved in 100 μl Laemmli buffer. 10 μl of each sample was run on an SDS-polyacrylamide gel (15%) as described [12].

2.4. *Purification of the mutated hTNF proteins*

The procedure described by Tavernier et al. [13] was followed, with the exception that the mutant proteins were only purified over a Mono-Q column (Pharmacia, Sweden), while the wild-type protein used as a control was further purified by means of an additional Mono-S chromatography step. Elution of the 28F and 114F mutants was controlled by a cytotoxicity assay [14] and/or by SDS-polyacrylamide gel electrophoresis.

2.5. *Determination of the specific activity*

An aliquot (2 μl) of the partially purified product was diluted in 800 μl H_2O and 200 μl of the Biorad protein dye reagent was added in order to measure the total protein concentration in a Beckmann DU-7 spectrophotometer. To calculate the concentrations of mutant proteins, their purity percentage was measured by running the mixture on a 15% SDS-polyacrylamide gel followed by scanning on a chromatogram densitometer CD-50 (Desaga, Heidelberg).

The cytotoxic activity of the samples was tested on L929 mouse fibroblast cells [14].

3. RESULTS

3.1. *Conversion of the position 28 and 114 tryptophan residues into phenylalanine by site-specific mutagenesis*

Using the selection method based on uracil-containing single-stranded templates of M13 DNA [10] for site-specific mutagenesis as described above, we generated 8% transitions to the Phe residue at position 28 and 50% at position 114, as tested by hybridization with the same ^{32}P-labeled oligonucleotide sequence as used for mutagenesis. These probes also generated a *Pst*I and a *Pvu*I restriction site in addition to the 28F mutation (28F probe, 26-mer); they also generated a *Sau*3A site and deleted a *Scr*FI as well as a *Bst*NI site adjacent to the 114F mutation (114F probe, 32-mer) (fig.3). As a result of this, we could simply monitor the presence of the mutations by restriction analysis.

The mutated genes were then transferred to the expression vector pMa and analysis of the resulting constructions pMa-T_4-hTNF28F-TA and pMa-T_4-hTNF114F-TA in *E. coli* MC1061 revealed mainly insoluble 28F and mainly soluble 114F mutant proteins, respectively, both constituting about 30% of the total protein content (fig.4).

3.2. *Purification of the mutant proteins*

Although the 28F mutation resulted in a mainly

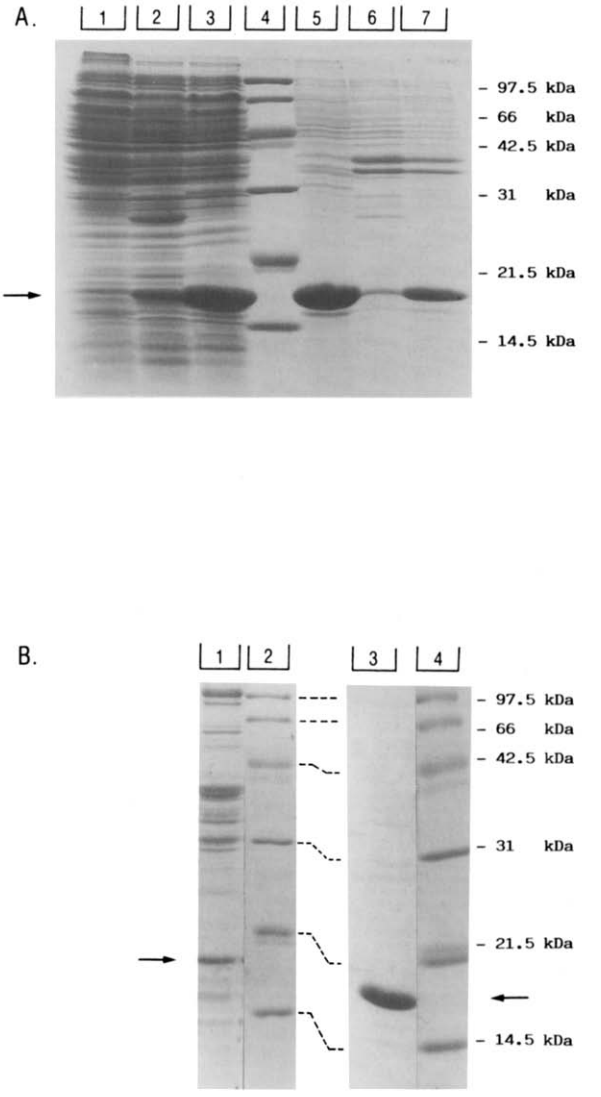

Fig.4. Expression of the 28F and 114F mutant proteins. Samples were prepared as described in section 2 and analyzed on a 15% SDS-polyacrylamide gel. (A: lysed cultures) Lanes: 1,5, hTNF 28F; 2,6, hTNF (wild-type); 3,7, hTNF 114F; 1–3, soluble fractions; lanes 5–7, insoluble fractions of the lysates; 4, Biorad molecular mass markers. (B: purified proteins) Lanes: 1, hTNF 28F; 2,4, Biorad molecular mass markers; 3, hTNF 114F.

insoluble TNF protein, the soluble fraction still contained sufficient biological activity to warrant purification. hTNF-28F eluted at 160 mM NaCl from the Mono Q column with a purity of about 12% relative to the total protein concentration of the fraction (fig.4). The eluted samples were tested for the presence of hTNF-28F in a cytotoxicity assay and checked for purity on an SDS-polyacrylamide gel.

The more soluble 114F mutant protein eluted at 280 mM NaCl from the Mono-Q column, with a purity of about 90% relative to the total protein concentration of the fraction, as judged by SDS-polyacrylamide gel electrophoresis (fig.4).

3.3. *Cytotoxic activity of the 28F and 114F mutant proteins*

The same samples as used for SDS-polyacrylamide gel electrophoresis were tested in an L929 cytotoxicity assay. This allowed us to determine the specific activity to be 8.5×10^6 and 5×10^6 U/μg for the 28F and 114F mutant TNF proteins, respectively. As the specific activity of the wild-type protein is about 2×10^7 U/mg [5], those of the 28F and the 114F mutant proteins can be estimated at 45 and 30%, respectively, of the wild-type activity.

4. DISCUSSION

We have shown that the replacement of either of the two highly conserved tryptophan residues in hTNF by phenylalanine does not markedly lower the cytotoxic activity of the molecule. Because tryptophan and phenylalanine have similar chemical characteristics, we hoped to cause minimal structural distortion in order to maintain the hTNF molecule in the native conformation. As observed for the 28F mutation, even a single conservative mutation is, however, sufficient to convert a soluble protein into a mainly insoluble form. Also, the 114F mutation seems to convert the wild-type protein, normally eluting from Mono Q at 140 mM NaCl, into a clearly different structure, eluting at 280 mM NaCl.

If the Trp residues were part of the active site responsible for cytotoxicity, one would expect that conversion into another amino acid, even with similar characteristics, would result in a drastic drop in cytolytic activity. Thus, it is likely that these two Trp residues play only a structural role, and that they perhaps are located sufficiently far from the active site that a local structural change does not interfere with cytotoxic activity. However, because the mutant proteins eluted on a gel filtration column (TSK, G 3000 SW, BRL) at the same position as the wild-type protein, it is unlikely that the Trp residues affect the oligomerization and that the slightly reduced activity should be due to a monomeric form. Also, these tryptophan residues might play an important role during the biosynthesis of TNF in eucaryotic cells or could affect post-translational events, such as secretion.

Similar results were obtained by Valenzuela et al. [15], who examined three amino acids in the human interferon-α protein, two of which were highly conserved in the different human interferon-α species and even the human interferon-β protein (intraspecies), and one was strictly conserved between man, mouse and livestock (interspecies).

Proteins mutated into these three amino acids did, however, maintain their antiviral activity. Pielak reported that the phylogenetically conserved phenylalanine 87 in yeast iso-1-cytochrome *c* is not directly involved in electron transfer, but performs a structural function by controlling the polarity of the heme environment [16]. Also, α-globin, mutated at the strongly conserved positions 127 and 141, seems to be functionally active, and persons carrying these mutations are asymptomatic [17,18].

So far, no satisfactory explanation for the conservation of the tryptophan residues in TNF and LT has been advanced. More insight into this phenomenon may be gained, however, when the two amino acids have been located on a three-dimensional model of the molecule. Crystals of hTNF have already been obtained and X-ray diffraction studies are in progress [19].

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REFERENCES

[1] Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V. and Markins, R.N. (1985) J. Biol. Chem. 260, 2345–2354.

[2] Beutler, B., Mahoney, J., Le Trang, N., Pekala, P. and Cerami, A. (1985) J. Exp. Med. 161, 984–995.

[3] Marmenout, A., Fransen, L., Tavernier, J., Van der Heyden, J., Tizard, R., Kawashima, E., Shaw, A., Johnson, M.J., Semon, D., Müller, R., Ruysschaert, M.R., Van Vliet, A. and Fiers, W. (1985) Eur. J. Biochem. 152, 515–522.

[4] Gray, P.W., Aggarwal, B.B., Benton, C.V., Bringman, T.S., Henzel, W.J., Jarret, J.A., Leung, D.W., Moffat, B., Ng, P., Svedersky, L.P., Palladino, M.A. and Nedwin, G.E. (1984) Nature 312, 721–724.

[5] Tavernier, J., Fransen, L., Marmenout, L., Van der Heyden, J., Müller, R., Ruysschaert, M.R., Van Vliet, A., Bauden, R. and Fiers, W. (1987) in: Lymphokines (Webb, D.R. and Goeddel, D.V. eds) vol.13, pp.181–198, Academic Press, New York.

[6] Gray, P.W., Chen, E., Li, C.B., Tang, W.L. and Ruddle, N. (1987) Nucleic Acids Res. 15, 3937.

[7] Hsu, Y.R., Narachi, M., Davis, J.M., Hennigan, P., Goldman, R.A., Geis, A., Carter, M., Stebbing, N., Alton, N.K. and Arakawa, T. (1986) Lymphokine Res. 5, suppl.1, 133–137.

[8] Davis, J.M., Narachi, M.A., Alton, N.K. and Arakawa, T. (1987) Biochemistry 26, 1322–1326.

[9] Narachi, A.N., Davis, J.M., Hsu, Y.R. and Arakawa, T. (1987) J. Biol. Chem. 262, 13107–13110.

[10] Kunkel, T.A. (1983) Proc. Natl. Acad. Sci. USA 82, 488–492.

[11] Stanssens, P., McKeown, Y., Friedrich, K. and Fritz, H.-J., in preparation.

[12] Laemmli, U.K. (1970) Nature 227, 680–685.

[13] Tavernier, J., Van Ostade, X., Hauquier, G., Bauden, R., Marmenout, A. and Fiers, W., in preparation.

[14] Ruff, M.R. and Gifford, G.E. (1981) in: Lymphokines (Pick, E. ed.) vol.2, pp.235–275, Academic Press, New York.

[15] Valenzuela, D., Weber, H. and Weissmann, C. (1985) Nature 313, 698–700.

[16] Pielak, G.J., Nauk, G. and Smith, N. (1985) Nature 313, 152–154.

[17] Vella, F. (1974) Biochim. Biophys. Acta 365, 318–322.

[18] Clegg, J.B., Weatherhall, D.J., Born, W.H. and Mustafa, D. (1969) Nature 222, 379–380.

[19] Lewit-Bentley, A., Fourme, R., Kahn, R., Prangé, T., Vachette, P., Tavernier, J., Hauquier, G. and Fiers, W. (1988) J. Mol. Biol. 199, 389–392.